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(54) Title: ISOLATION AND CHARACTERIZATION OF A *N. CRASSA* SILENCING GENE AND USES THEREOF

(57) Abstract: An isolated nucleic acid molecule encoding for a protein characterized in that it has a silencing activity and comprises a domain responsible for dsRNA interference is disclosed; furthermore expression vectors suitable for the expression of said sequence in bacteria, plants, animals and fungi are disclosed; the invention refers also to organisms transformed by such vectors.

ISOLATION AND CHARACTERIZATION OF A *N. CRASSA* SILENCING GENE AND USES THEREOF

The present invention relates to the isolation and characterization of a *Neurospora crassa* gene encoding for an essential activity in the co-suppression process and to uses and applications thereof in vegetal, animal and fungine fields.

The production of transgenic organisms is of large utility both in basic and applied biological research. The transgenic DNA is usually integrated in the genome and transferred as a Mendelian character. However, in various instances, the transgene introduction induces gene silencing phenomena (Flavell, R.B. 1994), i.e. the repression of the expression of the transgene itself and/or of one or more endogenous homologous genes.

The gene silencing (suppression of gene expression) can act at two levels: transcriptional (trans-inactivation) where transgenes contain sequences homologous to the silenced gene promoter (Vaucheret, 1993); and post-transcriptional (co-suppression) which requires homologies between coding regions (Flavell, 1994; Stam et al., 1997; Baulcombe, 1996).

Generally the silencing induced by a transgene requires an almost complete sequence homology (from 70% to 100%) between transgene and silenced gene sequences (Elkind, 1990).

In the *Neurospora crassa* filamentous fungus, during the vegetative phase, the presence of transgenes induces a post-transcriptional gene silencing phenomenon, named "quelling" (Cogoni et al., 1996).

By using the *al-1* gene (albino 1) (Schmidhauser et al., 1990) as silencing visual marker, many features of the phenomenon have been discovered (Cogoni et al., 1996). Particularly the *al-1* gene "quelling" in *Neurospora* is characterized in that: 1) the gene silencing is reversible further to the loss of transgene copies; 2) the reduction of mRNA basal level results from a post-transcriptional effect; 3) transgenes containing at least a region of 132 base pairs which is identical to the region encoding for the target gene are sufficient to induce the "quelling"; 4) the duplication of promoter sequences is ineffective to induce the silencing; 5) the "quelling" exhibits a dominant behavior in eterocarions containing both transgenic and untransformed nuclei, indicating the involvement of a trans-acting diffusible molecule among the nuclei; 6) the expression of an aberrant RNA transcribed by the transgenic locus is strictly correlated to silencing, suggesting that the "quelling" can be induced and/or mediated by a transgenic RNA molecule.

Therefore homologies between *Neurospora* silencing and plant co-suppression can be pointed out. The gene silencing in *Neurospora* is reversible, as result of transgenic copies instability during mitotic phase; in plants also the co-suppression reversion is associated with the reduction of transgene copy number, resulting from intra-chromosomal recombination during mitosis or meiosis (Mittelstein Scheid et al., 1994; Stam et al., 1997). Thus both in plants and in *Neurospora* the transgene presence is required to maintain the silencing. As in *Neurospora*, a decrease of the mRNA basal level of the silenced gene results from a post-transcriptional

mechanism (Dehio and Schell 1994; van Blokand et al., 1994; de Carvalho et al., 1995). Furthermore to induce the "quelling", transgenes must contain a portion of the silencing target gene coding sequence, being the promoter region ineffective. In plants coding regions with no promoter sequences can induce silencing (van Blokand et al., 1994) and, as in the "quelling", promoters or functionally active gene products are not required for the co-suppression.

One of the similarities between "quelling" and co-suppression in plants is that both mechanisms are mediated by diffusion factors. In *Neurospora* eterokaryotic strains, nuclei wherein the *albino-1* gene is silenced are able to induce the *al-1* gene silencing of the other not transformed nuclei, all sharing the same cytoplasmic environment (Cogoni et al., 1996). In plants the presence of a diffusion factor results from the fact that the co-suppression is effective in inhibiting the replication of Tobacco Etch Virus (TEV), a RNA virus with an exclusively cytoplasmic cycle. The occurrence of highly diffusible factors, which are effective to mediate the co-suppression, has been demonstrated using the grafting technique in tobacco (Palaqui et al., 1997), showing that silenced tobacco plants are able to transfer the silencing to non-silenced plants through grafting.

The fact that "quelling" and co-suppression share all these features suggests that mechanisms involved in post-transcriptional gene silencing in plants and in fungi can be evolved by an ancestral common mechanism.

Recently gene inactivation phenomena resulting from transgene introduction have been disclosed in animals. In *Drosophila melanogaster* the location of a transgene close

to heterochromatic centers results in a variegate expression (Wallrath and Elgin, 1995; Pirrotta, V., 1997). Similar expression profiles have been observed when the reference transgene is within tandem arrayed transposons, indicating that tandem repeats are effective to induce the chromatin condensation. (Dorer and Henikoff, 1994). Again in *Drosophila* Pal-Bhadra et al. (1997) have observed that the transgene introduction can lead to gene inactivation phenomena, similar to the co-suppression.

Gene silencing phenomena resulting from transegene sequence repeats have been disclosed recently in mammalians.

Garrick et al. (1998) produced mouse transgenic lines wherein 100 transgenic copies are present in a unique locus and are repeats-arrayed in direct tandem. The transgene expression has been disclosed to be inversely proportional to the number of occurring copies, indicating that silencing phenomena dependent on repeat copies are present also in mammalians.

It has been recently found that double stranded RNA molecules can induce a sequence-specific silencing in several organisms (Fire A., 1999). The mechanism known as dsRNAi (double stranded RNA interference) acts at a post-transcriptional level by inducing sequence-specific degradation of homologous mRNAs (Montgomery, Xu and Fire, 1998). Under this aspect, dsRNAi and quelling in *Neurospora* are similar mechanisms, both of them acting at a post-transcriptional level. In addition, both RNA-induced silencing and DNA-induced silencing can be transmitted from cell to cell.

Therefore the identification of *Neurospora* genes which are involved in the silencing is the first step to modulate the same process in plants, animals and fungi. The silencing modulation is of great relevance when transgenic organisms able to express the desired phenotype are produced.

The authors of the present invention have already isolated *Neurospora crassa* strains mutated at essential functions for gene silencing (Cogoni and Macino, 1997); 15 independent isolated mutants define three complementation groups, thus identifying the *qde-1, qde-2 and qde-3* genes (*qde* stands for "quelling"-deficient), whose products are essential to the silencing machinery. *qde* genes are essential to the *Neurospora* silencing, as suggested by the fact that silencing of three independent genes (*al-1, al-2* and *qa-2*) is impaired by *qde* mutations (Cogoni and Macino, 1997).

The authors of the present invention have already identified *qde-3* gene (PCT WO 00/327885) and *qde-1* gene (PCT WO 00/50581).

The authors of the invention have identified and cloned now one out of *Neurospora qde* genes, the *qde-2* gene, thus identifying one of required factors for silencing. By considering the similarity between "quelling" and co-suppression, genes orthologous to the isolated gene are involved in co-suppression and more generally in gene silencing in other organisms, like plants, fungi and animals.

The present invention can be applied with reference to two general scopes: 1) silencing potentiation as a tool for inactivating more effectively and durably a

desired gene, and 2) silencing suppression to obtain a better expression of the introduced transgenes.

The isolated *qde-2* gene can be introduced alone or with *qde-1* and/or *qde-3* genes in plants, animals or fungi, in order to inactivate the expression of selected genes. The aim is to activate a sequence-specific silencing mechanism both in deficient organisms and in organisms wherein the same is not very efficient. The gene silencing can be induced also by introducing specific double stranded DNA or RNA sequences, homologous to the gene to be inactivated.

As to the silencing potentiation, the over-expression of one or more genes controlling the phenomenon can lead to higher efficiency and/or stability thereof. Therefore the introduction of *qde-2* gene or of homologous genes thereof in organisms can constitute a tool to repress more effectively gene functions. Particularly this approach is specially useful in plants wherein the co-suppression is usually used for the "knock-out" of gene functions. In plants again the gene silencing potentiation can be used to obtain lines resistant to pathogen virus, by introducing transgenes encoding for viral sequences, in order to achieve the expression inhibition of the virus itself (Flavell et al., 1994).

Analogous applications are suitable for animals, wherein some indications suggest that silencing can inhibit the suitable expression of introduced transgenes (Garrick et al., 1998).

On the contrary, there are instances wherein it is desirable not to have or to reduce the gene silencing, i.e. where a transgene is to be over-expressed. It is

known that the co-suppression is strictly correlated both with the presence of an high copy number of the transgene, and with a transgene high expression. This correlation can hamper the production of transgenic organisms which express a transgene at high levels, because more high is the expression and/or the copy number, more probable is to evoke silencing responses. As above mentioned, analogous mechanisms of gene inactivation, dependent on a high copy number, have been disclosed in animals. In these circumstances plant or animal lines, totally or partially ineffective for silencing, constitute an ideal recipient wherein the desired gene can be over-expressed. The invention can be applied within this scope using different approaches:

A) Identification and production of mutant lines in genes homologous to *qde-2* gene, in plants, animals and fungi.

The identification of *Neurospora qde-2* gene, essential for silencing mechanism, can allow the isolation of mutant lines in other organisms, mutated in genes homologous to *qde-2*. For example by means of amplifications using degenerated primers, designed from the most conserved regions of *qde-2* gene, mutant lines in homologous genes can be identified, by analysis of insertion mutant gene banks, already available for many plant species. Both in fungi and animals such mutants can be obtained, following the identification of the homologous gene, by means of "gene disruption" techniques using homologous recombination.

B) Reduction of *qde-2* gene expression

Other strategies for the production of silencing-deficient lines comprise the use of *Neurospora qde-2* gene

or homologous genes thereof. *qde-2* or homologous genes can be introduced into suitable expression vectors to express them in an anti-sense orientation in order to inhibit the expression of resident endogenous genes. Alternatively portions of *qde-2* or of homologous genes can be over-expressed, in order to obtain a negative dominant effect and thus blocking the function of *qde-2* endogenous genes.

The authors of the present invention have cloned and characterised the *Neurospora crassa qde-2 gene*. The sequence analysis of the *qde-2* gene detected a region having a significant homology with the sequence of a *C. elegans* gene, *rde-1*, involved in the dsRNA mediated interference (Tabara et al., 1999).

The authors of the invention for the first time have demonstrated that the transgene induced post-transcriptional gene silencing and the dsRNA interference share common genetic mechanisms. This supports the hypothesis that the sequence specific gene silencing phenomena evolved from an ancestral mechanism aimed to protect the genome against transposons. Furthermore, the results of the authors suggest that dsRNA molecules are involved in the post-transcriptional gene silencing in fungi. dsRNA molecules could be produced directly from integrated trangenes as a result of the presence of inverted repeats or as an out come of transcription from convergent inverted promoters. Alternatively, single stranded aberrant RNA may be used as a template by an RNA-dependent RNA polymerase (such as QDE-1 protein) able to produce dsRNAs.

Within the scope of the invention the term homology is intended as similarity, i.e. number of identical

residues + number of conserved residues with respect to the total residues of the considered sequence.

Therefore it is an object of the present invention an isolated nucleic acid molecule encoding for a protein characterized in having a silencing activity and in comprising a domain responsible for dsRNA interference, wherein the domain is at least 25% homologous with the amino acid sequence from aa. 373 to aa. 910 of sequence in fig. 1 (SEQ ID No. 2). Preferably the domain is at least 30% homologous with the amino acid sequence from aa. 373 to aa. 910 of sequence in fig. 1 (SEQ ID No. 2). More preferably the domain is at least 38% homologous with the amino acid sequence from aa. 373 to aa. 910 of sequence in fig. 1 (SEQ ID No. 2). Most preferably the domain comprises the amino acid sequence from aa. 373 to aa. 910 of sequence in fig. 1 (SEQ ID No. 2). According to a particular embodiment the isolated nucleic acid molecule encodes for a protein having the amino acid sequence of fig. 1 (SEQ ID No. 2) or functional portions thereof. Even more preferably the isolated nucleic acid molecule has the sequence of fig. 1 (SEQ ID No. 1) or its complementary sequence.

A further object of the invention is an expression vector comprising, under the control of a promoter which directs the expression in bacteria, the isolated nucleic acid molecule of the invention. Those skilled in the art will appreciate that any plasmid suitable for a correct and effective expression of the protein of the expression in bacteria can be used and it is within the scope of the invention.

A further object of the invention is an expression vector comprising, under the control of a promoter which

directs the expression in plants or in specific plant organs, the isolated nucleic acid molecule of the invention, both in a sense and anti-sense orientation. Those skilled in the art will appreciate that any plasmid suitable for a correct and effective expression of the protein of the invention in plants or in specific plant organs can be used and it is within the scope of the invention.

A further object of the invention is an expression vector comprising, under the control of a promoter which directs the expression in fungi, the isolated nucleic acid molecule of the invention, both in a sense and anti-sense orientation. Those skilled in the art will appreciate that any plasmid suitable for a correct and effective expression of the inventive protein in fungi can be used and it is within the scope of the invention.

A further object of the invention is an expression vector comprising, under the control of a promoter which directs the expression in animals, the isolated nucleic acid molecule of the invention, both in a sense and anti-sense orientation. Those skilled in the art will appreciate that any plasmid suitable for a correct and effective expression of the protein of the invention in animals can be used and it is within the scope of the invention.

A further object of the invention is a prokaryotic organism transformed by using the expression vector active in bacteria of the invention.

A further object of the invention is a plant or a specific plant organ transformed by using the expression vector active in plants of the invention.

A further object of the invention is a plant mutated at the isolated nucleic acid molecule of the invention having a reduced or inhibited silencing activity.

A further object of the invention is a fungus transformed with the expression vector of the invention active in fungi.

A further object of the invention is a fungus mutated at the isolated nucleic acid molecule of the invention and having reduced or inhibited silencing activity.

A further object of the invention is a non-human animal transformed with the expression vector of the invention active in animals.

A further object of the invention is a non-human animal mutated at the isolated nucleic acid molecule of the invention and having a reduced or inhibited silencing activity.

A further object of the invention refers to a protein characterized in having a silencing activity and in comprising a domain responsible for dsRNA interference, wherein the domain is at least 25% homologous with the amino acid sequence from aa. 373 to aa. 910 in fig. 1 (SEQ ID No. 2). Preferably the domain is at least 30% homologous with the amino acid sequence from aa. 373 to aa. 910 in fig. 1 (SEQ ID No. 2). More preferably the domain is at least 38% homologous with the amino acid sequence from aa. 373 to aa. 910 in fig. 1 (SEQ ID No. 2). Most preferably the domain comprises the amino acid sequence from aa. 373 to aa. 910 in fig. 1 (SEQ ID No. 2). According to a particular embodiment the isolated nucleic acid molecule encodes for a protein

having the amino acid sequence of fig. 1 (SEQ ID No. 2) or functional portions thereof.

It is within the scope of the present invention the use of the isolated nucleic acid molecule of the invention to modulate gene silencing in plants, animals and fungi.

The present invention now will be described by way of non limiting examples with reference to the following figures:

Figure 1: The isolated nucleic acid molecule of the 5.7 Kb fragment containing the *qde-2* gene and flanking sequences (SEQ ID No.1). The amino acid sequence (SEQ ID No. 2) is shown above the nucleotide sequence.

Figure 2: It is schematically represented the pMXY2 plasmid insertion site, in the 80 mutant, used for insertional mutagenesis and consequent polimorphism of the restriction fragments by mean of DNA southern blot of a WT strain and of 80 and 820 mutant strains by using the entire restored flanking region as probe. The 820 mutant has a complete deletion of the *qde-2* gene.

Figure 3: Multiple alignment, at the conserved region, among *qde-2* and other proteins belonging to ago-eIF2C family: *A. thaliana ago-1*; rabbit *eIF2C*; *C. elegans rde-1*. Identical amino acids are shown in bold.

MATERIALS AND METHODS

E. coli strains

E. coli strain HB101 (F^-, hsdS20(rb^-, mb^-), supE44, recA13, ara14, proA2, rspL20(str^r), xyl-5) was used for cloning.

Neurospora crassa strains and growing conditions

Neurospora crassa following strains, supplied by Fungal Genetic Stock Center (FGSC, Dpt. Of Microbiology,

University of Kansas Medical Ctr. Kansas City, KA) were used:

- Wild type (FGSC 987);
- qa-2/aro9 (FGSC 3957A), (FGSC 3958a).

The 6XW strain (Cogoni et al., 1996) was obtained upon transformation of the FGCS 3958a strain with pX16 plasmid (Cogoni et al., 1996). This plasmid contains the *qa-2* gene used as selective marker and the *al-1* coding sequence.

The mutant strains M7, M20 (*qde-1*); M10, M11 (*qde-2*); M17, M18 (*qde-3*) are described in Cogoni and Macino, 1997.

The *qde* mutants were obtained by UV mutagenesis. As recipient the transforming strain (6xw) silenced at the *albino-1* gene was used. *qde* mutants were selected for their ability to recover a wild type unsilenced phenotype and then classified in three different complementation groups. By analyzing the *al-2* gene quelling frequency all of *qde* used mutants are defective for the general silencing mechanism.

Complementation assays with not forced heterocaryons were carried out according to Davis and DeSerres, 1970.

Plasmids and libraries

The plasmid pMXY2, disclosed in Campbell et al. 1994, used for insertional mutagenesis was obtained from Fungal Genetic Stock Center (FGSC, Dpt. Of Microbiology, University of Kansas Medical Ctr. Kansas City, KA). The plasmid contains the *Bml* gene (allele responsible of the benilate drug resistance), that was used as selective marker after transformation. The genomic DNA containing

the *qde-2* gene was isolated from a *N. Crassa* gene library in cosmids. (Cabibbo et al., 1991).

N. crassa transformation

Spheroplasts were prepared according to the Akins and Lambowitz (1985) protocol.

Southern Blot Analysis

Chromosomal DNA was prepared as disclosed by Irelan et al., 1993. 5 µg of genomic DNA were digested and blotted as reported in Maniatis et al.

DNA probes were: a) as to the *al-1* gene the probe is represented by a XbaI-ClaI restriction fragment of pX16 (Cogoni et al., 1996); b) as to the *BmI* gene the probe is represented by the 2.6Kb SalI fragment of pMXY2.

Northern Blot Analysis

N. crassa total RNA was extracted according to the protocol described by Cogoni et al., 1996. The mycelium was grown for two days at 30°C, then powdered in liquid nitrogen before RNA extraction. For Northern analysis 10 µg of RNA were formaldehyde denatured, electrophoresed on a 1% agarose, 7% formaldehyde gel, and blotted over Hybond N (Amersham) membranes. Hybridization was carried out in 50% formamide in the presence of ^{32}P labeled DNA probe $1.5x10^6$ cpm/ml.

RESULTS

Isolation of silencing mutant by insertional mutagenesis

Previously a *Neurospora* strain (6XW) wherein the *albino-1* resident gene was steadily silenced was used for UV mutagenisis that brought to the isolation of *qde* ("quelling" deficient) mutants in *N. crassa* induced gene silencing (Cogoni and Mancino 1997).

The 6XW strain shows an albino phenotype due to the lack of carotenoid biosynthesis, as results by the

silencing of the albino 1 gene expression (Schmidhauser et al., 1990). A mutation interfering with the silencing machinery is easily detectable by producing a wild type phenotype (bright orange) of the carotenoid biosynthesis. By means of complementation assays it was possible to establish that *qde* mutants belong to three complementation groups, indicating the presence of three genetic loci involved in the *Neurospora* silencing mechanism. In order to isolate the *qde* genes an insertional mutagenesis was carried out with the 6XW strain, previously used for UV mutagenesis. The insertional mutagenesis was carried out by transforming the 6XW strain with a plasmid, taking advantage of the fact that, after the transformation, plasmids are randomly inserted in the *Neurospora crassa* genome. The mutagenesis was carried out transforming the 6XW silenced strain with pMXY2 (see Materials and Methods) which contains the benilate resistance as selective marker. Transformed strains able to grow in the presence of benilate containing medium and showing a wild type phenotype for the carotenoid biosynthesis were selected. Out of 50.000 isolated independent transformed strains, a benilate resistant strain (80) was isolated, which showed the bright orange phenotype expected for a *qde* gene mutation. In order to verify that the silencing release was effectively due to a *qde* gene mutation and not to the loss of *al-1* transgene copies, the genomic DNA of the strain 80 was extracted and digested with SmaI and HindIII restriction enzymes. After blotting, DNA was hybridized with a probe corresponding to the coding sequence *of al-1*. The SmaI site is present only once in the *al-1* transgene containing plasmid and the digestion

by using said enzyme produces a 5.5Kb fragment corresponding to tandem arrayed *al-1* transgenes, while a 3.1Kb fragment is expected from the resident *al-1* locus. The number of *al-1* transgenic copies present in the 80 strain is comparable to that present in the silenced 6XW strain.

The strain 80 is mutated in *qde-2* gene

The strain 80 was assayed in a heterokaryon assay with a wild type strain and with M7, M20 (*qde-1*) M10, M11 (*qde-2*), M17, M18 (*qde-3*) mutants and with a wild strain (Cogoni and Macino, 1997). As shown in Table 1 the *al-1* gene silencing is restored producing an albino phenotype in all of heterocaryons but M10 and M11. This behavior is consistent with the presence of a *qde-2* gene recessive mutation in the strain 80.

Table 1

Reciprocal heterokaryons among the mutant 80 and previously characterized *qde* mutants.

	80	M7	M20	M10	M11	M17	M18
80	WT	AL	AL	WT	WT	AL	AL
M7		WT	WT	AL	AL	AL	AL
M20			WT	AL	AL	AL	AL
M10				WT	WT	AL	AL
M11					WT	AL	AL
M17						WT	WT
M18							WT

WT = heterokaryon with a wild type phenotype for carotenoid accumulation;

AL = heterokaryon with an albino phenotype wherein the *al-1* gene silencing is restored.

Recovery of sequences flanking the pMXY2 plasmid integration site

In order to recover sequences flanking the integration site or sites the following methodology was carried out. The genomic DNA of strain 80 was digested with Aat II enzyme. Subsequently the genomic DNA was ligated and the product used to transform *E. coli* cells that was screened in an ampicillin-containing medium. PQc1 plasmid was recovered and a DNA fragment containing sequences flanking the integration site was isolated from it by using Aat II and Cla I enzymes.

Isolation of genomic clones, their subcloning and complementation of the *qde-2* mutant

The fragment from pQc1 plasmid was used to probe a *Neurospora crassa* genomic library in cosmids. Three cosmids 6G10, 20C1 and 23F2 containing about 35 Kb genomic DNA inserts, were isolated. Such cosmids were used in transformation experiments of M11 and 80 mutants. All of cosmids are able to restore the *al-1* gene silencing in the two mutants, determining the appearance of an albino phenotype. The 20C1 cosmid was used to subclone a 5.7 Kb BamHI-BamHI fragment. This subclone was used for transformation experiments and resulted to be able to complement the *qde-2* phenotype, indicating that a *qde-2* functional gene is present in this plasmid.

Isolation and sequence of the *qde-2* cDNA

The sequence of BamHI-BamHI region allowed to deduce the amino acid sequence of the QDE-2 protein. The *qde-2* gene encodes for a 938 aa. putative protein (104 KDa). The genomic clone does not contain any introns since the reading frame does not contain any interruptions and intron acceptor and donor sequences were not identified (Fig. 1, Seq. ID No 1, 2).

The *qde-2* gene comprises an homologous domain with encoding genes for proteins that are responsible for dsRNA interference

The 938 aa sequence (SEQ ID No. 2) was used to search in database of amino acid sequences, by using the BLASTP algorithm. As showed in fig. 3, the search identified significant homologies with *argonaute-1* gene [with expected values (E value) of 2e-57] of *A. Thaliana* (mutants of this gene show developmental anomalies); *rde-1* gene [with expected values (E value) of 1e-23] of C. *elegans*, involved in gene silencing phenomena induced by double stranded RNA; *eIF2C* gene [with expected values (E value) of 5e-60] of rabbit isolated as an element belonging to transcription beginning complex.

Plant expression vector

The *qde-2* gene was inserted, in a sense orientation, into a vector containing a plant expression "cassette", including the 35S promoter and the PI-II "terminator" sequences. The vector also includes the *Streptomyces hygroscopicus bar* gene, which confers the phosphinotricine herbicide resistance to transformed plants. In an analogous vector to the above mentioned one, *qde-2* was inserted in an anti-sense orientation with respect to the 35S promoter.

The obtained vectors can be utilized to over-express the *qde-2* gene in plants, or to repress the gene expression of resident genes, which are homologous to *qde-2*.

Fungus expression vector

The *qde-2* gene was inserted in a vector containing a fungal specific expression "cassette", comprising the *A. nidulans trpC* gene promoter and terminator, both in a

sense and an anti-sense orientation. In addition the vector contains the bacterial *hph* gene, which confers the hygromicine drug resistance. The sense plasmid can be used to over express the *qde-2* gene, whereas the anti-sense plasmid is used to repress the expression of *qde-2* homologous genes in various fungine species.

Mammalian expression vector

The *qde-2* gene was inserted in a vector containing a mammalian specific expression "cassette", including the cytomegalovirus (CMV) promoter and SV40 termination and polyadenylation sequences both in a sense and anti-sense orientation. The vector includes also the neomicine phototransferase gene, as marker for mammalian cell selection. The sense plasmid can be used to over express the *qde-2* gene, whereas the anti-sense plasmid can be used to repress the expression of *qde-2* homologous genes in various mammalian species.

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Claims

1. An isolated nucleic acid molecule encoding for a protein characterized in having a silencing activity and in comprising a domain responsible for dsRNA interference, wherein the domain is at least 25% homologous with the amino acid sequence from aa. 373 to aa. 910 of SEQ ID No. 2.

2. An isolated nucleic acid molecule encoding for a protein characterized in having a silencing activity and comprising a domain responsible for dsRNA interference according to claim 1, wherein the domain is at least 30% homologous with the amino acid sequence from aa. 373 to aa. 910 of SEQ ID No. 2.

3. An isolated nucleic acid molecule encoding for a protein characterized in having a silencing activity and comprising a domain responsible for dsRNA interference according to claim 2, wherein the domain is at least 38% homologous with the amino acid sequence from aa. 373 to aa. 910 of SEQ ID No. 2.

4. An isolated nucleic acid molecule encoding for a protein characterized in having a silencing activity and comprising a domain responsible for dsRNA interference according to claim 3, wherein the domain is the amino acid sequence from aa. 373 to aa. 910 of SEQ ID No. 2.

5. An isolated nucleic acid molecule encoding for a protein characterized in having a silencing activity and comprising a domain responsible for dsRNA interference according to claim 4, wherein said isolated nucleic acid molecule encodes for a protein having the amino acid sequence of SEQ ID No. 2, or functional portions thereof.

6. An isolated nucleic acid molecule encoding for a protein characterized in having a silencing activity and comprising a domain responsible for dsRNA interference according to claim 5, wherein said isolated nucleic acid molecule has the sequence of SEQ ID No. 1 or its complementary sequence.

7. Expression vector comprising, under the control of a promoter that directs the expression in bacteria, the isolated nucleic acid molecule according to any one of claims 1-6.

8. Expression vector comprising, under the control of a promoter that directs the expression in plants or in specific plant organs, the isolated nucleic acid molecule according to any one of claims 1-6, both in a sense and anti-sense orientation.

9. Expression vector comprising, under the control of a promoter that directs the expression in fungi, the isolated nucleic acid molecule according to any one of claims 1-6 both in a sense and anti-sense orientation.

10. Expression vector comprising, under the control of a promoter that directs the expression in animals, the isolated nucleic acid molecule according to any one of claims 1-6 both in a sense and anti-sense orientation.

11. Prokaryotic organism transformed by using the expression vector active in bacteria according to claim 7.

12. Plants or a specific plant organ transformed by using the expression vector active in plants according to claim 8.

13. Plant mutated at the isolated nucleic acid molecule according to any one of claims 1-6 having a reduced or inhibited silencing activity.

14. Fungus transformed by using the expression vector active in fungi according to claim 9.

15. Fungus mutated at the isolated nucleic acid molecule according to any one of claims 1-6 having a reduced or inhibited silencing activity.

16. Non-human animal transformed by using the expression vector active in animals according to claim 10.

17. Non-human animal mutated at the isolated nucleic acid molecule according to any one of claims 1-6 having a reduced or inhibited silencing activity.

18. Protein characterized in having a silencing activity and comprising a domain responsible for dsRNA interference wherein the domain is at least 25% homologous to the amino acid sequence from aa. 373 to aa. 910 of SEQ ID No. 2.

19. Protein characterized in having a silencing activity and comprising a domain responsible for dsRNA interference according to claim 18 wherein the domain is at least 30% homologous to the amino acid sequence from aa. 373 to aa. 910 of SEQ ID No. 2.

20. Protein characterized in having a silencing activity and comprising a domain responsible for dsRNA interference according to claim 19 wherein the domain is at least 38% homologous to the amino acid sequence from aa. 373 to aa. 910 of SEQ ID No. 2.

21. Protein characterized in having a silencing activity and comprising a domain responsible for dsRNA interference according to claim 20 wherein the domain is the amino acid sequence from aa. 373 to aa. 910 of SEQ ID No. 2.

22. Protein characterized in having a silencing activity and comprising a domain responsible for dsRNA interference according to claim 21 comprising the amino acid sequence of SEQ ID No. 2 or functional portions thereof.

23. Use of the isolated nucleic acid molecule according to any one of claims 1-6 to modulate the gene silencing in plants, animals and fungi.

Length of cBAMqde2.txt: 5746 bp; Listed from: 1 to: 5746;
Translated from: 1039 to: 3852 (ORFs);
Genetic Code used: Universal; Lun, 27 ago 1956 18:50

Frame 1

```
          GGA TCC GCG TAG CAC ATC CTT TTC TTT TCC TTT TGG TTA TCC ATA ACC TTG GCA ACA CCT
                    9          18          27          36          45          54

 TTC TTT GCT TTC TCT CTC TTT TTC GCT TTA GAG ACC TAC GCA ACT ACC CAT CAT CAT TTT CTG ATA
           69          78          87          96         105         114         123

 TCG ACA TAT CAC CCA ACA ACA TCA TCA TCA TCT ACT ACC AGT AAT CCC GCA TCG GAG GAG TAG TCG
          135         144         153         162         171         180         189

 TTT CGC TCG ATT ACT CTT TTT TTT GCG TCC GGA GTG CGA CAA AGT AGC GGC TTA TAA CAA GTC CAA
          201         210         219         228         237         246         255

 GTT GGA AAA AAA CCA TCA ATC AGT GGT ATT TCT CTC TTG GCA AAT CCA CAA CAA TCC CCT TCC ACG
          267         276         285         294         303         312         321

 ACA AAC AAA CAA ACA ACC TAC CTT AAC TAT CCT CTT GCT TAC CTA CGT ACC TGC CTA CCT ACC TAC
          333         342         351         360         369         378         387

 CTA CCT ACC TAC CTC TGC TCA ACC AAC CAT CTC GTC AAT CAA ACC GAA CCG AAC CAA ACC GAA CGA
          399         408         417         426         435         444         453

 TAG CCG AAT AAG CTC TCG TGC CTT GTT GCT CTA CTC GAC AAT CTG TTA CCA CCA ACA CTA CAA GTT
          465         474         483         492         501         510         519

 TAA CAG TCA TGT CTG ACA ATC GTG GCG GTC GTG GAG GTC GTG GCG GCG GTG GTC GCG GCG GCG GCG
          531         540         549         558         567         576         585

 GCG GCG GCG GAG GCC GTG GAG GTG GTC AGC AAG GCG GCG GTG GAG GCC GTG GAG GTG GTT ACC AAG
          597         606         615         624         633         642         651

 GCA GCG GCG GCG GTG GAG GCC GTG GCG GCG GTT ATC AAG GCG GTG GCG GCG GTG ACC GTG GAG GCC
          663         672         681         690         699         708         717

 GTG GCG GCG GTT ATC AAG GCG GTG GTG GCG GTG GTT TCC AAG GCG GCG GTG GAA GGG GTG GCC GTG
          729         738         747         756         765         774         783

 GCG GCG GTT TCC AAG GCG GCG GCG GCG GCG GCC GTG GTG GCT TCG GCG GAG GAC AGG GCG CGG GAG
          795         804         813         822         831         840         849

 GAT ACG AAC CCC CTC CAC CGG ATG TCT ACA AGT AGG TGC CTC TCC ATT TTT TTT TAC CAT TCA ACA
          861         870         879         888         897         906         915

 TGA TGC TGA CAC GAC TTT AGG GGA ATT GAC GGT CGT GGT GCC CCC GAG CCT GAC GCC CAG ATC ACC
          927         936         945         954         963         972         981

                                                                    M   S   K   L
 AAA CTC GAG GAT GAT TGG ATC AAG AAG CAC GTC AGC GAC AAT CTG GTC ACT TCC ATG AGC AAG CTT
          993        1002        1011        1020        1029        1038        1047

 S   L   S   E   K   E   K   A   N   N   L   P   V   R   P   G   H   G   T   M   G   E
 TCG CTC AGC GAG AAG GAG AAA GCC AAC AAC TTG CCG GTT CGC CCT GGC CAT GGT ACC ATG GGC GAG
         1059        1068        1077        1086        1095        1104        1113

 K   V   K   L   W   A   N   Y   F   K   I   N   I   K   S   P   A   I   Y   R   Y   T
 AAG GTG AAG CTT TGG GCC AAC TAT TTC AAA ATC AAC ATC AAA TCA CCA GCC ATT TAC AGG TAC ACC
         1125        1134        1143        1152        1161        1170        1179

 I   K   V   A   A   T   E   E   K   L   G   K   E   A   E   V   A   S   K   K   V   E
 ATC AAA GTT GCC GCC ACC GAG GAA AAG CTC GGA AAG GAA GCT GAG GTC GCA TCC AAG AAA GTG GAG
         1191        1200        1209        1218        1227        1236        1245

 V   V   V   G   K   L   L   K   Q   I   E   A   N   V   K   S   V   A   I   A   S   D
 GTG GTG GTT GGG AAA CTG CTC AAG CAG ATC GAA GCC AAC GTG AAA TCC GTG GCG ATT GCC AGC GAT
         1257        1266        1275        1284        1293        1302        1311
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FIG. 1-1

```
F  K  V  H  L  V  T  T  T  K  L  K  V  P  E  N  R  I  F  E  V  T
TTC AAA GTG CAC CTG GTG ACG ACC ACC AAG CTC AAA GTT CCC GAG AAC CGC ATC TTT GAG GTG ACG
      1323       1332       1341       1350       1359       1368       1377

W  T  E  P  S  S  N  Q  N  L  P  S  K  P  Q  T  W  V  V  K  V  E
TGG ACC GAG CCG AGT TCC AAC CAA AAC CTG CCC AGC AAG CCC CAG ACT TGG GTG GTC AAG GTG GAG
      1389       1398       1407       1416       1425       1434       1443

E  S  V  E  T  C  D  F  G  K  V  L  N  E  L  T  T  L  D  P  K  L
GAG AGT GTC GAA ACC TGC GAT TTC GGC AAG GTG CTG AAC GAG CTC ACG ACA CTT GAT CCC AAG CTC
      1455       1464       1473       1482       1491       1500       1509

D  G  D  F  P  K  Y  N  V  E  L  D  A  L  N  T  I  V  T  H  H  A
GAC GGA GAC TTT CCC AAG TAC AAT GTG GAG CTC GAT GCC CTC AAC ACC ATT GTG ACT CAT CAT GCC
      1521       1530       1539       1548       1557       1566       1575

R  A  D  D  N  V  A  V  V  G  R  G  R  F  F  A  I  G  D  D  L  I
CGC GCC GAC GAC AAT GTT GCG GTG GTG GGA AGG GGA AGG TTT TTT GCC ATT GGT GAT GAC CTC ATT
      1587       1596       1605       1614       1623       1632       1641

E  Q  V  R  P  H  D  S  P  L  V  I  L  R  G  Y  F  A  S  V  R  P
GAA CAA GTG CGG CCC CAT GAC TCC CCT TTG GTC ATC TTG CGA GGA TAT TTT GCC AGC GTC CGT CCA
      1653       1662       1671       1680       1689       1698       1707

A  T  G  R  L  L  L  N  T  N  I  T  H  G  V  F  R  P  G  V  K  L
GCT ACC GGA AGA CTT TTA CTC AAT ACC AAC ATC ACG CAT GGT GTC TTC CGT CCT GGG GTC AAA CTT
      1719       1728       1737       1746       1755       1764       1773

A  Q  L  F  Q  E  L  G  L  D  V  M  D  K  C  N  A  W  N  E  V  T
GCA CAG CTG TTT CAG GAA CTT GGA CTT GAC GTA ATG GAC AAA TGC AAT GCC TGG AAC GAA GTA ACC
      1785       1794       1803       1812       1821       1830       1839

K  N  Q  L  N  D  K  M  R  R  V  H  K  V  L  A  K  G  R  V  E  L
AAA AAT CAG CTC AAC GAC AAG ATG CGC AGA GTT CAC AAG GTC CTG GCT AAG GGC CGT GTC GAG TTG
      1851       1860       1869       1878       1887       1896       1905

N  A  P  F  L  I  D  G  K  I  V  Y  K  K  C  Y  R  T  L  N  G  I
AAT GCC CCA TTC CTT ATT GAT GGA AAG ATT GTT TAT AAA AAA TGT TAC CGC ACG CTC AAT GGC ATT
      1917       1926       1935       1944       1953       1962       1971

A  N  R  G  D  E  R  G  K  Q  K  D  G  K  E  V  R  Y  P  P  L  F
GCT AAC CGT GGC GAC GAA AGG GGG AAG CAA AAG GAT GGT AAA GAA GTC CGA TAT CCG CCC TTG TTC
      1983       1992       2001       2010       2019       2028       2037

G  I  P  G  V  Q  V  G  G  P  T  S  C  Q  F  Y  L  R  A  R  E  T
GGG ATT CCG GGT GTC CAG GTT GGC GGC CCG ACC TCT TGT CAG TTC TAC TTG CGT GCG CGA GAG ACA
      2049       2058       2067       2076       2085       2094       2103

K  D  G  A  A  P  P  P  T  P  G  L  P  S  N  A  Y  I  T  V  A  N
AAG GAT GGC GCT GCC CCT CCT CCG ACT CCC GGC CTG CCG AGC AAC GCG TAC ATC ACG GTA GCG AAC
      2115       2124       2133       2142       2151       2160       2169

Y  Y  K  Q  R  Y  G  I  T  A  N  A  S  L  P  L  V  N  V  G  T  K
TAT TAT AAA CAA CGG TAC GGA ATA ACC GCC AAT GCT TCG CTT CCT CTG GTC AAC GTT GGC ACC AAG
      2181       2190       2199       2208       2217       2226       2235

E  K  A  I  Y  V  L  A  E  F  C  T  L  V  K  G  R  S  V  K  A  K
GAA AAG GCG ATT TAC GTC TTG GCC GAG TTT TGT ACG CTG GTC AAA GGC CGT TCC GTC AAG GCT AAG
      2247       2256       2265       2274       2283       2292       2301

L  T  A  N  E  A  D  N  M  I  K  F  A  C  R  A  P  S  L  N  A  Q
CTG ACA GCC AAC GAG GCG GAC AAC ATG ATT AAG TTT GCT TGC AGA GCT CCT TCG CTG AAC GCT CAG
      2313       2322       2331       2340       2349       2358       2367

S  I  V  T  K  G  R  Q  T  L  G  L  D  K  S  L  T  L  G  K  F  K
TCT ATC GTG ACG AAA GGC AGA CAG ACA CTT GGT CTT GAT AAA AGC CTG ACG CTT GGC AAG TTC AAG
      2379       2388       2397       2406       2415       2424       2433

V  S  I  D  K  E  L  I  T  V  V  G  R  E  L  K  P  P  M  L  T  Y
GTT TCG ATC GAC AAG GAG CTG ATC ACC GTT GTC GGG CGT GAG CTC AAG CCT CCG ATG CTT ACG TAC
      2445       2454       2463       2472       2481       2490       2499
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FIG. 1-2

```
S   G   N   K   T   V   E   P   Q   D   G   G   W   L   M   K   F   V   K   V   A   R
AGC GGT AAC AAG ACG GTA GAG CCG CAG GAC GGC GGG TGG TTG ATG AAG TTT GTC AAG GTC GCC AGA
        2511        2520        2529        2538        2547        2556        2565

P   C   R   K   I   E   K   W   T   Y   L   E   L   K   G   S   K   A   N   E   G   V
CCT TGC CGC AAG ATT GAG AAG TGG ACA TAC TTG GAA CTG AAG GGT TCC AAG GCA AAC GAA GGG GTG
        2577        2586        2595        2604        2613        2622        2631

P   Q   A   M   T   A   F   A   E   F   L   N   R   T   G   I   P   I   N   P   R   F
CCG CAA GCT ATG ACC GCT TTT GCC GAA TTC TTG AAC AGA ACG GGC ATC CCG ATT AAC CCC AGG TTC
        2643        2652        2661        2670        2679        2688        2697

S   P   G   M   S   M   S   V   P   G   S   E   K   E   F   F   A   K   V   K   E   L
TCG CCG GGC ATG AGC ATG TCA GTT CCA GGG AGC GAA AAA GAG TTC TTT GCC AAA GTG AAG GAA CTC
        2709        2718        2727        2736        2745        2754        2763

M   S   S   H   Q   F   V   V   V   L   L   P   R   K   D   V   A   I   Y   N   M   V
ATG AGC TCG CAC CAA TTT GTG GTG GTT CTT TTA CCC AGA AAG GAT GTT GCG ATC TAC AAT ATG GTG
        2775        2784        2793        2802        2811        2820        2829

K   R   A   A   D   I   T   F   G   V   H   T   V   C   C   V   A   E   K   F   L   S
AAG CGG GCT GCC GAT ATC ACA TTT GGC GTT CAC ACA GTC TGT TGT GTA GCC GAA AAG TTC CTT AGC
        2841        2850        2859        2868        2877        2886        2895

T   K   G   Q   L   G   Y   F   A   N   V   G   L   K   V   N   L   K   F   G   G   T
ACT AAG GGG CAG CTG GGG TAT TTT GCC AAC GTC GGC CTC AAG GTC AAC CTC AAG TTT GGC GGC ACC
        2907        2916        2925        2934        2943        2952        2961

N   H   N   I   K   T   P   I   P   L   L   A   K   G   K   T   M   V   V   G   Y   D
AAT CAC AAT ATC AAG ACG CCC ATT CCT TTG CTC GCC AAG GGG AAG ACG ATG GTG GTG GGC TAT GAT
        2973        2982        2991        3000        3009        3018        3027

V   T   H   P   T   N   L   A   A   G   Q   S   P   A   S   A   P   S   I   V   G   L
GTC ACC CAT CCG ACC AAT CTA GCG GCT GGA CAA TCG CCT GCA TCG GCT CCC AGT ATT GTC GGC CTG
        3039        3048        3057        3066        3075        3084        3093

V   S   T   I   D   Q   H   L   G   Q   W   P   A   M   V   W   N   N   P   H   G   Q
GTC TCA ACC ATC GAC CAA CAC CTT GGA CAA TGG CCT GCA ATG GTT TGG AAC AAC CCG CAC GGC CAG
        3105        3114        3123        3132        3141        3150        3159

E   S   M   T   E   Q   F   T   D   K   F   K   T   R   L   E   L   W   R   S   N   P
GAG TCC ATG ACG GAA CAG TTT ACG GAC AAG TTC AAG ACG CGT CTG GAA CTA TGG CGC AGC AAT CCC
        3171        3180        3189        3198        3207        3216        3225

A   N   N   R   S   L   P   E   N   I   L   I   F   R   D   G   V   S   E   G   Q   F
GCA AAC AAC CGC AGT CTC CCC GAG AAT ATC CTG ATT TTC CGC GAT GGC GTC TCC GAG GGA CAG TTC
        3237        3246        3255        3264        3273        3282        3291

Q   M   V   I   K   D   E   L   P   L   V   R   A   A   C   K   L   V   Y   P   A   G
CAG ATG GTC ATC AAG GAC GAG CTA CCC CTG GTT CGC GCC GCC TGC AAG CTG GTG TAT CCA GCT GGC
        3303        3312        3321        3330        3339        3348        3357

K   L   P   R   I   T   L   I   V   S   V   K   R   H   Q   T   R   F   F   P   T   D
AAG CTA CCG CGT ATT ACG CTG ATT GTC TCT GTC AAG CGC CAC CAG ACT CGC TTC TTC CCA ACG GAC
        3369        3378        3387        3396        3405        3414        3423

P   K   H   I   H   F   K   S   K   S   P   K   E   G   T   V   V   D   R   G   V   T
CCG AAG CAT ATT CAC TTC AAG TCC AAG AGC CCC AAG GAG GGT ACT GTG GTT GAC CGC GGC GTG ACC
        3435        3444        3453        3462        3471        3480        3489

N   V   R   Y   W   D   F   F   L   Q   A   H   A   S   L   Q   G   T   A   R   S   A
AAC GTC CGC TAT TGG GAC TTC TTT TTG CAG GCG CAC GCG TCG CTC CAG GGC ACG GCC CGC TCG GCT
        3501        3510        3519        3528        3537        3546        3555

H   Y   T   V   L   V   D   E   I   F   R   A   D   Y   G   N   K   A   A   D   T   L
CAC TAC ACA GTT CTG GTG GAT GAG ATT TTC AGG GCC GAC TAT GGA AAC AAG GCG GCC GAC ACG CTG
        3567        3576        3585        3594        3603        3612        3621

E   Q   L   T   H   D   M   C   Y   L   F   G   R   A   T   K   A   V   S   I   C   P
GAG CAG CTG ACG CAT GAC ATG TGT TAT CTC TTT GGA CGA GCC ACC AAG GCT GTC AGT ATC TGC CCG
```

FIG. 1-3

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      3633        3642        3651        3660        3669        3678        3687

P   A   Y   Y   A   D   L   V   C   D   R   A   R   I   H   Q   K   E   L   F   D   A
CCT GCG TAC TAT GCC GAC TTG GTG TGC GAC CGG GCG CGT ATC CAT CAG AAG GAG CTC TTT GAC GCC
      3699        3708        3717        3726        3735        3744        3753

L   D   E   N   D   S   V   K   T   D   D   F   A   R   W   G   N   S   G   A   V   H
CTC GAT GAA AAC GAT AGC GTT AAG ACC GAT GAT TTC GCA AGA TGG GGT AAC TCC GGG GCT GTT CAT
      3765        3774        3783        3792        3801        3810        3819

P   N   L   R   N   S   M   Y   Y   I
CCC AAC CTT AGG AAC TCC ATG TAC TAT ATC TAG GCT TGT CAA TTG TGT GCT GGA ATG TAC TGG AGC
      3831        3840        3849        3858        3867        3876        3885

ATA TAA GTG ACG CGA TGG AAG CCT AAT CGT CTC TGA ATA TGG ATC AAA GAC AGC GTT TGC TTT TTC
      3897        3906        3915        3924        3933        3942        3951

GGG GCT TCT AGT TTC TAC AGC GAT TTG TGT GGA TTG TTT CTT GTT CTG TTT CTT GGT TCT TTC TTT
      3963        3972        3981        3990        3999        4008        4017

CTT TTT TTT GTG TCT CTG TCT GCC TTT GTA CTG CAT GCA AAC GTG CAC TCT GAA TGA TGA ACG ACA
      4029        4038        4047        4056        4065        4074        4083

CCA TTT GAC GAT TGG ATA AGA GAT GAC AGA CTG CAG ATA CTA TCA TGC GCA ATG GAA AAC ACG AAC
      4095        4104        4113        4122        4131        4140        4149

AAC CAA GGT TTT TGA TTC CTT CAA TAG CGA AAT ATA GAA AAA GAA ACA AAA AAA AAA ACA ACA ACA
      4161        4170        4179        4188        4197        4206        4215

AAT AAT GGA AGT ATG ATT AAA CAC ATT GAG CGC GAT GAC TGA CTG GTG TTG TGA ATG GCG TGT TGG
      4227        4236        4245        4254        4263        4272        4281

TTT TCT TCT TTC TTG AAA ATT TAG AAC CGT AAA TGT TAT ATC ATG TGA TGT AAT GTA ATA ACA TAT
      4293        4302        4311        4320        4329        4338        4347

TTA TAT CTC GTT GTA TTC TTG TAC ACA CTT TCC AGG ATA ACA TGG TCT GAC ATG GTA TTT CTG ACG
      4359        4368        4377        4386        4395        4404        4413

TAC AAA AAA GAA AAA GAA AAA CAG GAA ACC ATG AAC CCG CGA CAA AGC TGT TCC AGT TGT TAC AAT
      4425        4434        4443        4452        4461        4470        4479

GAT GAT GAT GAT GAT GAC CTA CTA CCT AAG GTA TTC TAT CTT AGC CAA GGT ATT CTC TCG CAT CCT
      4491        4500        4509        4518        4527        4536        4545

ATT CCA TCC TAT CCT AAC CCG AGC CTA ACC CGA GCC TAA ATA CCT AAA CTC CTA AAC TCC TTA ACT
      4557        4566        4575        4584        4593        4602        4611

CCT TAA CTC CTT TCT AAA TGT CTA AAC CCC CAA ACT ATG AGA CGA CCC GAA CCC GAA ACC CTA ATA
      4623        4632        4641        4650        4659        4668        4677

AAA GTA TTT ATA AAC CAT CAT AAA AGA AAA AAA ACC ATC ATA CAT GGA TGA TCA AAA CAA ACA GAA
      4689        4698        4707        4716        4725        4734        4743

ACG GAA ACA ACA CAA CCA GCT ACC CGC TCA AGA CTT TCA TTC GTT AAT TCA TCA CTC ACT CAC TCA
      4755        4764        4773        4782        4791        4800        4809

CTC ACT CAC TCA GCA GCA AAA TAC CGT TTT GTC CTG CTA TTC GTT TGT TGC GCC TTG ATT TCA GGC
      4821        4830        4839        4848        4857        4866        4875

GGG ACA ATG GTG TGA TGT ACG ACG TGG GGG CGG TAG ACT GCG TCT ACT GGT GGC ATC CTT TAC AAT
      4887        4896        4905        4914        4923        4932        4941

TTT TTA GTG TGT CAG TAT GTG ATG TAT TCA ATG CTA TTG AAC TGA GGG GGG CTG ATG GAT AGT GGG
      4953        4962        4971        4980        4989        4998        5007

GAG AGA ACA CCT GAC GGA TAG AGG GAA GGA ACT GGA CGC CTG GGG GGA AGT GAG AGA GGG GGA TGG
      5019        5028        5037        5046        5055        5064        5073

TGG GGA ATA GAT GAA AAG AGA AGA GGA GTG AGA GCA CAA GAA GAA AGA ATG AAT GTT GGT GAC AAA
      5085        5094        5103        5112        5121        5130        5139
```

FIG. 1-4

```
GTT AAA GAA AAG GAA GGG GGG AAA GAG AAG AGG ACA GGT GTG GTG AGT GAA TTG AGT GAA AGG AAG
      5151        5160        5169        5178        5187        5196        5205

GGA AAA AAC GGA GAA GGA AAA AAA AAA CAT AAA AAA AAA AAA AAA AAC AGA AAG AAA GAA CTA ACC
      5217        5226        5235        5244        5253        5262        5271

AAT CAT CCA AAC TCA GCG GAA AGT ACT CAT ACA AAA GGT CGG CTG CCT CAA TCG GAC TCC CCA CAT
      5283        5292        5301        5310        5319        5328        5337

TCT CTT TCT GGT ACT GAT TCT GCT GCC CCA GAC TTC CAC TTT CAA AGT GGC TAT CAC CCT TAT TGT
      5349        5358        5367        5376        5385        5394        5403

TGT TAG AGT GAG TAG TAG ACG TAA GTC CTC CCG ATC CGG AGC CAA AAC CCA TCC CTT TCC CAG CTG
      5415        5424        5433        5442        5451        5460        5469

TAT CCC TCT TCA ATC CAC CAG TAG CAA CAC CCA TCT TGC CAT AGA GCG GAC TAT CCC CTG CCC CTG
      5481        5490        5499        5508        5517        5526        5535

CCC CTG CCG AGC CAG GAG TAG CAG TCC TAT TCA TAG GCG GAC TCC TCT GCT CGT CTT CCG ACA GGG
      5547        5556        5565        5574        5583        5592        5601

ACA AAC TAA TTG GTA GGG CAC CCG CAG CAG AGG AGG AGG TAT TTC TGT GAT GAC TGG TTC TGT TTG
      5613        5622        5631        5640        5649        5658        5667

GGG CAG CTA AGG GCG TGG GTT TCC TTC GTG AGC CGC TGT TGT GAT TGT TGG CGG CGG CGT CCG AGG
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ATA AGG ATC C
      5745
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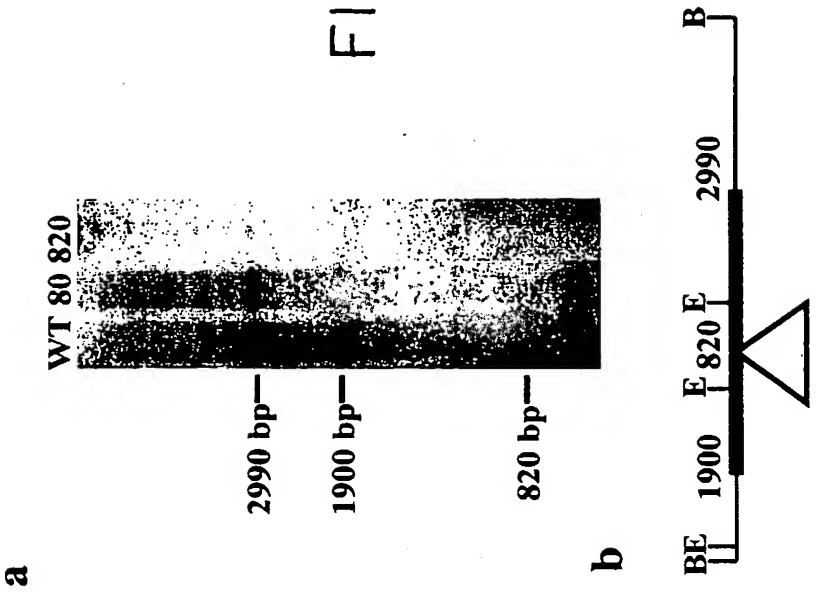
FIG. 1-5

FIG. 2



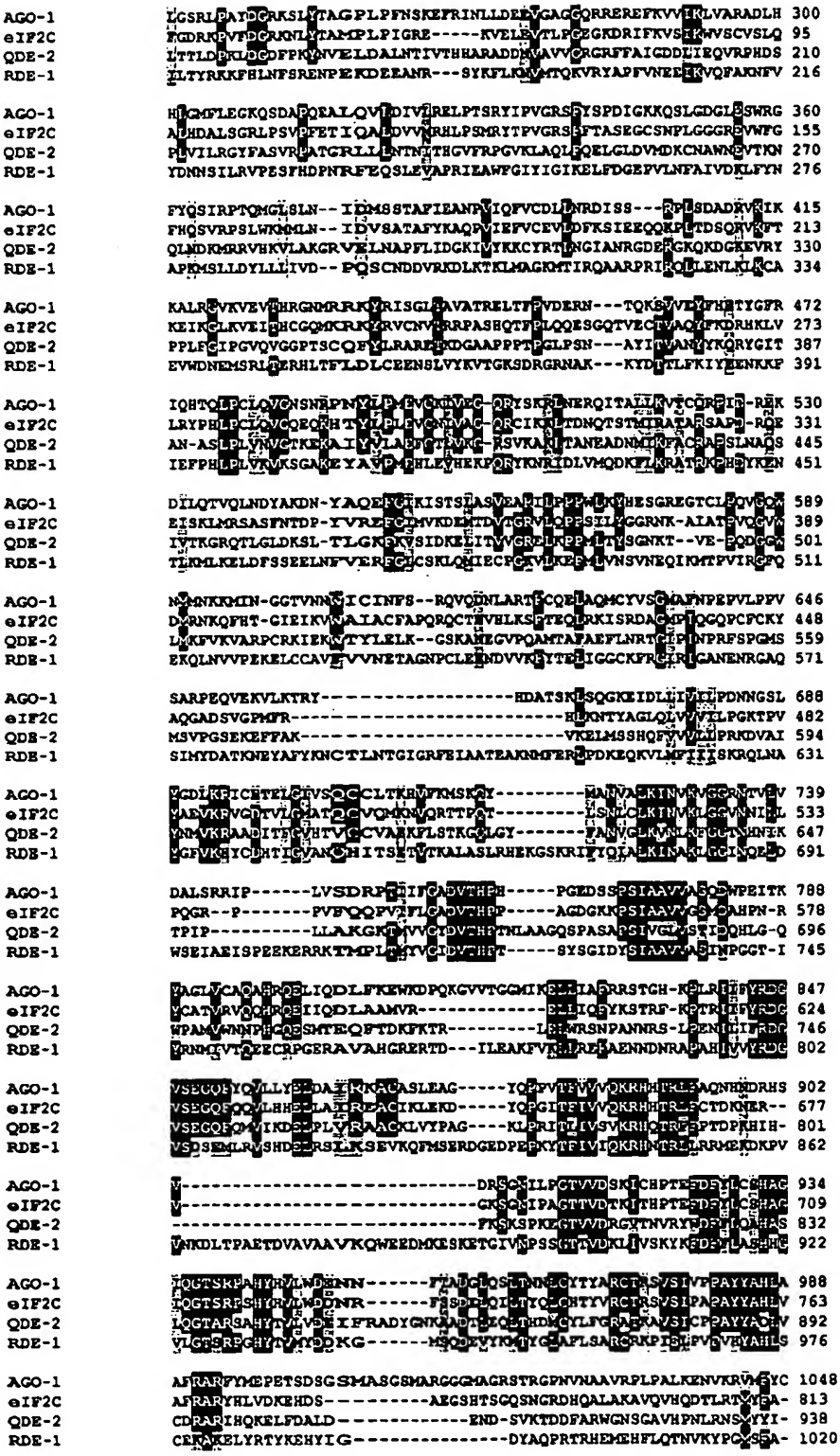


FIG. 3

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Macino, Giuseppe
Catalanotto, Caterina
Azzalin, Gianluca

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cccgagcctg acgcccagat caccaaactc gaggatgatt ggatcaagaa gcacgtcagc 1020

gacaatctgg tcacttcc atg agc aag ctt tcg ctc agc gag aag gag aaa   1071
                  Met Ser Lys Leu Ser Leu Ser Glu Lys Glu Lys
                    1               5                  10

gcc aac aac ttg ccg gtt cgc cct ggc cat ggt acc atg ggc gag aag   1119
Ala Asn Asn Leu Pro Val Arg Pro Gly His Gly Thr Met Gly Glu Lys
            15                  20                  25

gtg aag ctt tgg gcc aac tat ttc aaa atc aac atc aaa tca cca gcc   1167
Val Lys Leu Trp Ala Asn Tyr Phe Lys Ile Asn Ile Lys Ser Pro Ala
        30                  35                  40

att tac agg tac acc atc aaa gtt gcc gcc acc gag gaa aag ctc gga   1215
Ile Tyr Arg Tyr Thr Ile Lys Val Ala Ala Thr Glu Glu Lys Leu Gly
    45                  50                  55

aag gaa gct gag gtc gca tcc aag aaa gtg gag gtg gtg gtt ggg aaa   1263
Lys Glu Ala Glu Val Ala Ser Lys Lys Val Glu Val Val Val Gly Lys
60                  65                  70                  75

ctg ctc aag cag atc gaa gcc aac gtg aaa tcc gtg gcg att gcc agc   1311
Leu Leu Lys Gln Ile Glu Ala Asn Val Lys Ser Val Ala Ile Ala Ser
                80                  85                  90

gat ttc aaa gtg cac ctg gtg acg acc acc aag ctc aaa gtt ccc gag   1359
Asp Phe Lys Val His Leu Val Thr Thr Thr Lys Leu Lys Val Pro Glu
            95                  100                 105

aac cgc atc ttt gag gtg acg tgg acc gag ccg agt tcc aac caa aac   1407
```

```
Asn Arg Ile Phe Glu Val Thr Trp Thr Glu Pro Ser Ser Asn Gln Asn
        110                 115                 120

ctg ccc agc aag ccc cag act tgg gtg gtc aag gtg gag gag agt gtc   1455
Leu Pro Ser Lys Pro Gln Thr Trp Val Val Lys Val Glu Glu Ser Val
    125                 130                 135

gaa acc tgc gat ttc ggc aag gtg ctg aac gag ctc acg aca ctt gat   1503
Glu Thr Cys Asp Phe Gly Lys Val Leu Asn Glu Leu Thr Thr Leu Asp
140                 145                 150                 155

ccc aag ctc gac gga gac ttt ccc aag tac aat gtg gag ctc gat gcc   1551
Pro Lys Leu Asp Gly Asp Phe Pro Lys Tyr Asn Val Glu Leu Asp Ala
                160                 165                 170

ctc aac acc att gtg act cat cat gcc cgc gcc gac gac aat gtt gcg   1599
Leu Asn Thr Ile Val Thr His His Ala Arg Ala Asp Asp Asn Val Ala
            175                 180                 185

gtg gtg gga agg gga agg ttt ttt gcc att ggt gat gac ctc att gaa   1647
Val Val Gly Arg Gly Arg Phe Phe Ala Ile Gly Asp Asp Leu Ile Glu
        190                 195                 200

caa gtg cgg ccc cat gac tcc cct ttg gtc atc ttg cga gga tat ttt   1695
Gln Val Arg Pro His Asp Ser Pro Leu Val Ile Leu Arg Gly Tyr Phe
    205                 210                 215

gcc agc gtc cgt cca gct acc gga aga ctt tta ctc aat acc aac atc   1743
Ala Ser Val Arg Pro Ala Thr Gly Arg Leu Leu Leu Asn Thr Asn Ile
220                 225                 230                 235

acg cat ggt gtc ttc cgt cct ggg gtc aaa ctt gca cag ctg ttt cag   1791
Thr His Gly Val Phe Arg Pro Gly Val Lys Leu Ala Gln Leu Phe Gln
                240                 245                 250

gaa ctt gga ctt gac gta atg gac aaa tgc aat gcc tgg aac gaa gta   1839
Glu Leu Gly Leu Asp Val Met Asp Lys Cys Asn Ala Trp Asn Glu Val
            255                 260                 265

acc aaa aat cag ctc aac gac aag atg cgc aga gtt cac aag gtc ctg   1887
Thr Lys Asn Gln Leu Asn Asp Lys Met Arg Arg Val His Lys Val Leu
        270                 275                 280

gct aag ggc cgt gtc gag ttg aat gcc cca ttc ctt att gat gga aag   1935
Ala Lys Gly Arg Val Glu Leu Asn Ala Pro Phe Leu Ile Asp Gly Lys
    285                 290                 295

att gtt tat aaa aaa tgt tac cgc acg ctc aat ggc att gct aac cgt   1983
```

```
Ile Val Tyr Lys Lys Cys Tyr Arg Thr Leu Asn Gly Ile Ala Asn Arg
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ggc gac gaa agg ggg aag caa aag gat ggt aaa gaa gtc cga tat ccg   2031
Gly Asp Glu Arg Gly Lys Gln Lys Asp Gly Lys Glu Val Arg Tyr Pro
                320                 325                 330

ccc ttg ttc ggg att ccg ggt gtc cag gtt ggc ggc ccg acc tct tgt   2079
Pro Leu Phe Gly Ile Pro Gly Val Gln Val Gly Gly Pro Thr Ser Cys
            335                 340                 345

cag ttc tac ttg cgt gcg cga gag aca aag gat ggc gct gcc cct cct   2127
Gln Phe Tyr Leu Arg Ala Arg Glu Thr Lys Asp Gly Ala Ala Pro Pro
        350                 355                 360

ccg act ccc ggc ctg ccg agc aac gcg tac atc acg gta gcg aac tat   2175
Pro Thr Pro Gly Leu Pro Ser Asn Ala Tyr Ile Thr Val Ala Asn Tyr
    365                 370                 375

tat aaa caa cgg tac gga ata acc gcc aat gct tcg ctt cct ctg gtc   2223
Tyr Lys Gln Arg Tyr Gly Ile Thr Ala Asn Ala Ser Leu Pro Leu Val
380                 385                 390                 395

aac gtt ggc acc aag gaa aag gcg att tac gtc ttg gcc gag ttt tgt   2271
Asn Val Gly Thr Lys Glu Lys Ala Ile Tyr Val Leu Ala Glu Phe Cys
                400                 405                 410

acg ctg gtc aaa ggc cgt tcc gtc aag gct aag ctg aca gcc aac gag   2319
Thr Leu Val Lys Gly Arg Ser Val Lys Ala Lys Leu Thr Ala Asn Glu
            415                 420                 425

gcg gac aac atg att aag ttt gct tgc aga gct cct tcg ctg aac gct   2367
Ala Asp Asn Met Ile Lys Phe Ala Cys Arg Ala Pro Ser Leu Asn Ala
        430                 435                 440

cag tct atc gtg acg aaa ggc aga cag aca ctt ggt ctt gat aaa agc   2415
Gln Ser Ile Val Thr Lys Gly Arg Gln Thr Leu Gly Leu Asp Lys Ser
    445                 450                 455

ctg acg ctt ggc aag ttc aag gtt tcg atc gac aag gag ctg atc acc   2463
Leu Thr Leu Gly Lys Phe Lys Val Ser Ile Asp Lys Glu Leu Ile Thr
460                 465                 470                 475

gtt gtc ggg cgt gag ctc aag cct ccg atg ctt acg tac agc ggt aac   2511
Val Val Gly Arg Glu Leu Lys Pro Pro Met Leu Thr Tyr Ser Gly Asn
                480                 485                 490

aag acg gta gag ccg cag gac ggc ggg tgg ttg atg aag ttt gtc aag   2559
```

```
Lys Thr Val Glu Pro Gln Asp Gly Gly Trp Leu Met Lys Phe Val Lys
            495                 500                 505

gtc gcc aga cct tgc cgc aag att gag aag tgg aca tac ttg gaa ctg   2607
Val Ala Arg Pro Cys Arg Lys Ile Glu Lys Trp Thr Tyr Leu Glu Leu
        510                 515                 520

aag ggt tcc aag gca aac gaa ggg gtg ccg caa gct atg acc gct ttt   2655
Lys Gly Ser Lys Ala Asn Glu Gly Val Pro Gln Ala Met Thr Ala Phe
    525                 530                 535

gcc gaa ttc ttg aac aga acg ggc atc ccg att aac ccc agg ttc tcg   2703
Ala Glu Phe Leu Asn Arg Thr Gly Ile Pro Ile Asn Pro Arg Phe Ser
540                 545                 550                 555

ccg ggc atg agc atg tca gtt cca ggg agc gaa aaa gag ttc ttt gcc   2751
Pro Gly Met Ser Met Ser Val Pro Gly Ser Glu Lys Glu Phe Phe Ala
                560                 565                 570

aaa gtg aag gaa ctc atg agc tcg cac caa ttt gtg gtg gtt ctt tta   2799
Lys Val Lys Glu Leu Met Ser Ser His Gln Phe Val Val Val Leu Leu
            575                 580                 585

ccc aga aag gat gtt gcg atc tac aat atg gtg aag cgg gct gcc gat   2847
Pro Arg Lys Asp Val Ala Ile Tyr Asn Met Val Lys Arg Ala Ala Asp
        590                 595                 600

atc aca ttt ggc gtt cac aca gtc tgt tgt gta gcc gaa aag ttc ctt   2895
Ile Thr Phe Gly Val His Thr Val Cys Cys Val Ala Glu Lys Phe Leu
    605                 610                 615

agc act aag ggg cag ctg ggg tat ttt gcc aac gtc ggc ctc aag gtc   2943
Ser Thr Lys Gly Gln Leu Gly Tyr Phe Ala Asn Val Gly Leu Lys Val
620                 625                 630                 635

aac ctc aag ttt ggc ggc acc aat cac aat atc aag acg ccc att cct   2991
Asn Leu Lys Phe Gly Gly Thr Asn His Asn Ile Lys Thr Pro Ile Pro
                640                 645                 650

ttg ctc gcc aag ggg aag acg atg gtg gtg ggc tat gat gtc acc cat   3039
Leu Leu Ala Lys Gly Lys Thr Met Val Val Gly Tyr Asp Val Thr His
            655                 660                 665

ccg acc aat cta gcg gct gga caa tcg cct gca tcg gct ccc agt att   3087
Pro Thr Asn Leu Ala Ala Gly Gln Ser Pro Ala Ser Ala Pro Ser Ile
        670                 675                 680

gtc ggc ctg gtc tca acc atc gac caa cac ctt gga caa tgg cct gca   3135
```

```
Val Gly Leu Val Ser Thr Ile Asp Gln His Leu Gly Gln Trp Pro Ala
    685                 690                 695

atg gtt tgg aac aac ccg cac ggc cag gag tcc atg acg gaa cag ttt   3183
Met Val Trp Asn Asn Pro His Gly Gln Glu Ser Met Thr Glu Gln Phe
700                 705                 710                 715

acg gac aag ttc aag acg cgt ctg gaa cta tgg cgc agc aat ccc gca   3231
Thr Asp Lys Phe Lys Thr Arg Leu Glu Leu Trp Arg Ser Asn Pro Ala
                720                 725                 730

aac aac cgc agt ctc ccc gag aat atc ctg att ttc cgc gat ggc gtc   3279
Asn Asn Arg Ser Leu Pro Glu Asn Ile Leu Ile Phe Arg Asp Gly Val
            735                 740                 745

tcc gag gga cag ttc cag atg gtc atc aag gac gag cta ccc ctg gtt   3327
Ser Glu Gly Gln Phe Gln Met Val Ile Lys Asp Glu Leu Pro Leu Val
        750                 755                 760

cgc gcc gcc tgc aag ctg gtg tat cca gct ggc aag cta ccg cgt att   3375
Arg Ala Ala Cys Lys Leu Val Tyr Pro Ala Gly Lys Leu Pro Arg Ile
    765                 770                 775

acg ctg att gtc tct gtc aag cgc cac cag act cgc ttc ttc cca acg   3423
Thr Leu Ile Val Ser Val Lys Arg His Gln Thr Arg Phe Phe Pro Thr
780                 785                 790                 795

gac ccg aag cat att cac ttc aag tcc aag agc ccc aag gag ggt act   3471
Asp Pro Lys His Ile His Phe Lys Ser Lys Ser Pro Lys Glu Gly Thr
                800                 805                 810

gtg gtt gac cgc ggc gtg acc aac gtc cgc tat tgg gac ttc ttt ttg   3519
Val Val Asp Arg Gly Val Thr Asn Val Arg Tyr Trp Asp Phe Phe Leu
            815                 820                 825

cag gcg cac gcg tcg ctc cag ggc acg gcc cgc tcg gct cac tac aca   3567
Gln Ala His Ala Ser Leu Gln Gly Thr Ala Arg Ser Ala His Tyr Thr
        830                 835                 840

gtt ctg gtg gat gag att ttc agg gcc gac tat gga aac aag gcg gcc   3615
Val Leu Val Asp Glu Ile Phe Arg Ala Asp Tyr Gly Asn Lys Ala Ala
    845                 850                 855

gac acg ctg gag cag ctg acg cat gac atg tgt tat ctc ttt gga cga   3663
Asp Thr Leu Glu Gln Leu Thr His Asp Met Cys Tyr Leu Phe Gly Arg
860                 865                 870                 875

gcc acc aag gct gtc agt atc tgc ccg cct gcg tac tat gcc gac ttg   3711
```

```
Ala Thr Lys Ala Val Ser Ile Cys Pro Pro Ala Tyr Tyr Ala Asp Leu
                880                 885                 890

gtg tgc gac cgg gcg cgt atc cat cag aag gag ctc ttt gac gcc ctc   3759
Val Cys Asp Arg Ala Arg Ile His Gln Lys Glu Leu Phe Asp Ala Leu
            895                 900                 905

gat gaa aac gat agc gtt aag acc gat gat ttc gca aga tgg ggt aac   3807
Asp Glu Asn Asp Ser Val Lys Thr Asp Asp Phe Ala Arg Trp Gly Asn
        910                 915                 920

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Ser Gly Ala Val His Pro Asn Leu Arg Asn Ser Met Tyr Tyr Ile
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Ala Ser Lys Lys Val Glu Val Val Val Gly Lys Leu Leu Lys Gln Ile
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Glu Ala Asn Val Lys Ser Val Ala Ile Ala Ser Asp Phe Lys Val His
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Leu Val Thr Thr Thr Lys Leu Lys Val Pro Glu Asn Arg Ile Phe Glu
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Val Thr Trp Thr Glu Pro Ser Ser Asn Gln Asn Leu Pro Ser Lys Pro
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Asp Ser Pro Leu Val Ile Leu Arg Gly Tyr Phe Ala Ser Val Arg Pro
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Ala Thr Gly Arg Leu Leu Leu Asn Thr Asn Ile Thr His Gly Val Phe
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Glu Leu Asn Ala Pro Phe Leu Ile Asp Gly Lys Ile Val Tyr Lys Lys
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Cys Tyr Arg Thr Leu Asn Gly Ile Ala Asn Arg Gly Asp Glu Arg Gly

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Lys Gln Lys Asp Gly Lys Glu Val Arg Tyr Pro Pro Leu Phe Gly Ile
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Pro Gly Val Gln Val Gly Gly Pro Thr Ser Cys Gln Phe Tyr Leu Arg
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Ala Arg Glu Thr Lys Asp Gly Ala Ala Pro Pro Pro Thr Pro Gly Leu
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Pro Ser Asn Ala Tyr Ile Thr Val Ala Asn Tyr Tyr Lys Gln Arg Tyr
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Gly Ile Thr Ala Asn Ala Ser Leu Pro Leu Val Asn Val Gly Thr Lys
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Glu Lys Ala Ile Tyr Val Leu Ala Glu Phe Cys Thr Leu Val Lys Gly
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Arg Ser Val Lys Ala Lys Leu Thr Ala Asn Glu Ala Asp Asn Met Ile
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Lys Phe Ala Cys Arg Ala Pro Ser Leu Asn Ala Gln Ser Ile Val Thr
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Asn Glu Gly Val Pro Gln Ala Met Thr Ala Phe Ala Glu Phe Leu Asn
530 535 540

Arg Thr Gly Ile Pro Ile Asn Pro Arg Phe Ser Pro Gly Met Ser Met
545 550 555 560

Ser Val Pro Gly Ser Glu Lys Glu Phe Phe Ala Lys Val Lys Glu Leu

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                565                 570                 575

Met Ser Ser His Gln Phe Val Val Val Leu Leu Pro Arg Lys Asp Val
            580                 585                 590

Ala Ile Tyr Asn Met Val Lys Arg Ala Ala Asp Ile Thr Phe Gly Val
        595                 600                 605

His Thr Val Cys Cys Val Ala Glu Lys Phe Leu Ser Thr Lys Gly Gln
    610                 615                 620

Leu Gly Tyr Phe Ala Asn Val Gly Leu Lys Val Asn Leu Lys Phe Gly
625                 630                 635                 640

Gly Thr Asn His Asn Ile Lys Thr Pro Ile Pro Leu Leu Ala Lys Gly
                645                 650                 655

Lys Thr Met Val Val Gly Tyr Asp Val Thr His Pro Thr Asn Leu Ala
            660                 665                 670

Ala Gly Gln Ser Pro Ala Ser Ala Pro Ser Ile Val Gly Leu Val Ser
        675                 680                 685

Thr Ile Asp Gln His Leu Gly Gln Trp Pro Ala Met Val Trp Asn Asn
    690                 695                 700

Pro His Gly Gln Glu Ser Met Thr Glu Gln Phe Thr Asp Lys Phe Lys
705                 710                 715                 720

Thr Arg Leu Glu Leu Trp Arg Ser Asn Pro Ala Asn Asn Arg Ser Leu
                725                 730                 735

Pro Glu Asn Ile Leu Ile Phe Arg Asp Gly Val Ser Glu Gly Gln Phe
            740                 745                 750

Gln Met Val Ile Lys Asp Glu Leu Pro Leu Val Arg Ala Ala Cys Lys
        755                 760                 765

Leu Val Tyr Pro Ala Gly Lys Leu Pro Arg Ile Thr Leu Ile Val Ser
    770                 775                 780

Val Lys Arg His Gln Thr Arg Phe Phe Pro Thr Asp Pro Lys His Ile
785                 790                 795                 800

His Phe Lys Ser Lys Ser Pro Lys Glu Gly Thr Val Val Asp Arg Gly
                805                 810                 815

Val Thr Asn Val Arg Tyr Trp Asp Phe Phe Leu Gln Ala His Ala Ser
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          820                 825                 830

Leu Gln Gly Thr Ala Arg Ser Ala His Tyr Thr Val Leu Val Asp Glu
        835                 840                 845

Ile Phe Arg Ala Asp Tyr Gly Asn Lys Ala Ala Asp Thr Leu Glu Gln
    850                 855                 860

Leu Thr His Asp Met Cys Tyr Leu Phe Gly Arg Ala Thr Lys Ala Val
865                 870                 875                 880

Ser Ile Cys Pro Pro Ala Tyr Tyr Ala Asp Leu Val Cys Asp Arg Ala
            885                 890                 895

Arg Ile His Gln Lys Glu Leu Phe Asp Ala Leu Asp Glu Asn Asp Ser
            900                 905                 910

Val Lys Thr Asp Asp Phe Ala Arg Trp Gly Asn Ser Gly Ala Val His
        915                 920                 925

Pro Asn Leu Arg Asn Ser Met Tyr Tyr Ile
    930                 935
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